

The three isoenzymes of human inositol-1,4,5-trisphosphate 3-kinase show specific intracellular localization but comparable Ca^{2+} responses on transfection in COS-7 cells

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Inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] 3-kinase catalyses the phosphorylation of InsP_3 to inositol 1,3,4,5-tetrakisphosphate. cDNAs encoding three human isoenzymes of InsP_3 3-kinase (A, B and C) have been reported previously [Choi, Kim, Lee, Moon, Sim, Kim, Chung and Rhee (1990) *Science* **248**, 64–66; Dewaste, Pouillon, Moreau, Shears, Takazawa and Erneux (2000) *Biochem. J.* **352**, 343–351; Dewaste, Roymans, Moreau and Erneux (2002) *Biochem. Biophys. Res. Commun.* **291**, 400–405; Takazawa, Perret, Dumont and Erneux (1991) *Biochem. Biophys. Res. Commun.* **174**, 529–535]. The localization of InsP_3 3-kinase isoenzymes fused at their N-terminus to the green fluorescent protein has been studied by confocal microscopy. The A isoform appeared to associate with the cytoskeleton, whereas the C isoform was totally cytoplasmic. The B isoform had a more complex localization: it appeared in the plasma membrane, cytoskeleton and in the endoplasmic reticulum. The three human

isoenzymes of InsP_3 3-kinase can thus be distinguished by their N-terminal sequence, sensitivity to Ca^{2+} /calmodulin and localization on transfection in COS-7 cells. We have compared the cytosolic Ca^{2+} responses induced by ATP in COS-7 cells transfected with the three isoenzymes. Cells expressing high levels of any of the three isoforms no longer respond to ATP, whereas cells expressing low levels of each enzyme showed a reduced response consisting of one to three Ca^{2+} spikes in response to 100 μM ATP. These effects were seen only in wild-type InsP_3 3-kinase-transfected cells. 3-Kinase-dead mutant cells behaved as vector-transfected cells. The results highlight the potential role of the three isoforms of InsP_3 3-kinase as direct InsP_3 metabolizing enzymes and direct regulators of Ca^{2+} responses to extracellular signals.

Key words: inositol 1,4,5-trisphosphate 3-kinase, intracellular calcium, isoenzyme localization.

INTRODUCTION

Hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C produces the second messengers inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol, which take part in the mobilization of intracellular Ca^{2+} and activation of protein kinase C respectively [1]. InsP_3 is metabolized by type I InsP_3 5-phosphatase to produce inositol 1,4-bisphosphate, which is inactive in Ca^{2+} mobilization [2]. In addition, InsP_3 can be phosphorylated to inositol 1,3,4,5-tetrakisphosphate (InsP_4) by an InsP_3 3-kinase reaction [3]. Depending on the cell type, different effects of InsP_4 have been reported, some of which are directly or indirectly related to Ca^{2+} homeostasis [4,5]. In perfused lacrimal cells and in permeabilized mouse lymphoma cells, synergistic effects of InsP_4 on InsP_3 -stimulated Ca^{2+} release have been reported [6,7]. In HeLa cells, electroporation of InsP_4 causes a transient increase in the frequency of Ca^{2+} oscillations in response to histamine [8]. A high-affinity InsP_4 -binding protein has been purified from pig platelets. The cDNA encoding this putative receptor encoded a GTPase-activating protein. *In vitro*, it shows GTPase-activating protein activity against Ras that is inhibited by phospholipids and specifically stimulated by InsP_4 [9,10]. This protein is associated constitutively with the plasma membrane [11]. The possible interaction between InsP_4 and

type I InsP_3 5-phosphatase has been investigated in RBL cells: InsP_4 facilitates store-operated Ca^{2+} influx by inhibition of type I inositol 5-phosphatase. This results in the protection of InsP_3 against hydrolysis to an inactive inositol phosphate [12]. Recent results indicate that remodelling of endoplasmic reticulum (ER) in mouse lacrimal acinar cells could be a physiological regulator of Ca^{2+} signalling and propose a role for InsP_4 in the control of this process. In this model, InsP_4 potentiates InsP_3 -induced Ca^{2+} release [13]. Finally, InsP_3 3-kinase reaction could be a pathway in the production of $\text{Ins}(1,3,4)\text{P}_3$ and subsequent phosphorylation to InsP_5 and InsP_6 [14,15].

Many cell types respond to agonists acting via the phosphoinositide signalling pathway by the generation of a complex Ca^{2+} response and oscillations [1,16–18]. The InsP_3 receptors are responsible, at least partially, for this complex behaviour being controlled directly by Ca^{2+} , calmodulin (CaM), phosphorylation and interaction with protein partners [19]. Another control mechanism may involve the metabolism of InsP_3 and/or InsP_4 . Type I InsP_3 5-phosphatase is prenylated to the plasma membrane providing a mechanism of concentrating this activity to a restricted area of the cell [20]. Previous studies using transfected CHO-K1 (where CHO stands for Chinese-hamster ovary) cell lines support the contention that the ATP-induced increase in InsP_3 concentration is restricted essentially to the site of its production

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} ; CaM, calmodulin; CHO, Chinese-hamster ovary; ER, endoplasmic reticulum; GFP, green fluorescent protein; InsP_3 , inositol 1,4,5-trisphosphate; InsP_4 , inositol 1,3,4,5-tetrakisphosphate; for brevity the one-letter system for amino acids has been used, K264A, for example, means $\text{Lys}^{264} \rightarrow \text{Ala}$.

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near the plasma membrane, where it can be metabolized by the type I InsP_3 5-phosphatase. This enzyme controls directly the cytosolic Ca^{2+} ($[\text{Ca}^{2+}]_i$) response in intact cells [21].

The mammalian InsP_3 3-kinases consist of a family of multiple isoenzymes referred to as InsP_3 3-kinases A, B and C [22–25]. The three isoenzymes share a relatively well-conserved catalytic domain and specifically convert InsP_3 into InsP_4 . The N-terminal end of the rat A isoform targets the enzyme to the filamentous actin-rich dendritic spines in neurons [26]. The results suggest a specific role of this isoenzyme related to these targeting properties, particularly in hippocampal neurons and dendritic spines. Truncated rat InsP_3 3-kinase B was reported to be localized both at the ER and in the cytosol in rat liver [27]. No results have been reported yet for the localization of the human C isoform.

Full-length sequences of human InsP_3 3-kinases A–C have been reported before [23–25]. In the present study, we aimed to compare the properties of the three human isoenzymes under similar experimental conditions. We have compared the Ca^{2+} responses induced by ATP in COS-7 cells transfected with the three isoenzymes, wild-type and catalytic mutants, fused at their N-terminus to the green fluorescent protein (GFP). Successfully transfected cells could be recognized by GFP fluorescence of the cells and used directly to measure Ca^{2+} signals. We also compared the localization of each inositol kinase isoform in cells. The results highlight the potential role of the three isoforms of InsP_3 3-kinase as direct InsP_3 -metabolizing enzymes and direct regulators of the Ca^{2+} responses in response to extracellular signals. Any change in the V_{\max} values of the InsP_3 3-kinase reaction could therefore influence the Ca^{2+} response.

EXPERIMENTAL

Materials and InsP_3 3-kinase enzymic assay

InsP_3 was obtained from Sigma. FuGENE 6 transfection reagent was from Roche Molecular Biochemicals. $[\text{^3H}]\text{InsP}_3$ (22 Ci/mmol) was from Dupont-NEN-PerkinElmer (Zaventem, Belgium). Dowex 1-X8 (formate form) was from Bio-Rad Laboratories. Rhodamine phalloidin was from Molecular Probes. pDsRed2-ER, the C-terminal-enhanced fluorescent protein vectors pEGFP-C3 and -C1 were from Clontech Laboratories (Erembodegem, Belgium). The cDNA clones of InsP_3 3-kinase A in pBluescript, InsP_3 3-kinase B in pcDNA3 and InsP_3 3-kinase C in pcDNA3-His were reported previously [23–25]. ATP, UTP, Pefabloc and leupeptin were from Pentapharm-Roche Diagnostics (Vilvoorde, Belgium) and ICN Biomedicals (Asse-Relegem, Belgium). Cell culture media and antibiotics were from Gibco BRL. Anti-GFP antibodies (A-6455) were from Molecular Probes. QuikChange XL Site-Directed Mutagenesis kit was from Stratagene. InsP_3 3-kinase enzymic assay at 10 μM InsP_3 substrate level was reported earlier [28], except that after incubation the reaction was terminated by adding 1 ml of ice-cold 0.4 M ammonium formate/0.1 M formic acid and separation performed immediately on 0.5 ml Dowex 1-X8 columns. Our assays were conducted in triplicates using three different dilutions of the lysate to ensure linearity with respect to protein concentrations. We also used 2.5 mM bisphosphoglycerate to prevent any degradation of the substrate or product during incubation.

Subcloning of InsP_3 3-kinases A–C in pEGFP-C1 and -C3

The InsP_3 3-kinase A coding sequence in pBluescript was subcloned using the *EcoRI* restriction site of pEGFP-C3 expression vector. This InsP_3 3-kinase A plasmid was referred to as GFP–

InsP_3 3-kinase A plasmid. It was digested with *XhoI*–*KpnI* to obtain the N-terminal-deleted plasmid encoding InsP_3 3-kinase A stretching from amino acid residues 133 to 461. The InsP_3 3-kinase B coding sequence in pcDNA3 was subcloned using the *HindIII* restriction site of pEGFP-C3 resulting in GFP– InsP_3 3-kinase B plasmid. This InsP_3 3-kinase B plasmid was digested with *BglIII*–*KpnI* to obtain the N-terminal-deleted plasmid encoding InsP_3 3-kinase B stretching from amino acid residues 483 to 946. The InsP_3 3-kinase C coding sequence in pcDNA3-His was subcloned using the *BamHI* restriction site of pEGFP-C1 resulting in GFP– InsP_3 3-kinase C plasmid.

Site-directed mutagenesis

Site-directed mutagenesis was performed on GFP 3-kinase A, B and C isoforms by using the QuikChange XL Site-Directed Mutagenesis kit. Briefly, forward primers were designated according to the manufacturer's instructions as follows: for the A isoform K264A, 5'-GTGTGCTCGACTGCGCAATGGGCGTCAGGAC-3'; for the B isoform D897N, 5'-GTGTGGATGATCAACTTTGGGAAAACACGCCCC-3'; and for the C isoform K486A, 5'-CCATTATGGACTGCGCGATGGGCAGCAGGACC-3'. Reverse primers were the complementary sequences of the forward primers. PCR conditions, parental template digestion and transformation into *Escherichia coli* strain XL10-Gold were performed according to the manufacturer's instructions.

Expression of InsP_3 3-kinase isoenzymes in COS-7 and CHO-K1 cells

The full-length coding regions of InsP_3 3-kinase isoenzymes in pEGFP were transfected in COS-7 or CHO-K1 cells using FuGENE according to the manufacturer's instructions. For the fluorescence analysis, we used 10^5 cells/dish of 3 cm diameter and 1.5 μg of DNA/dish. For Ca^{2+} measurements, we used 5×10^4 cells/2 cm² chamber slides and 0.5 μg of DNA. The COS-7 cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C and maintained in Dulbecco's modified Eagle's medium supplemented with 1% sodium pyruvate, 2% (v/v) penicillin/streptomycin, 1% fungizone and 10% (v/v) foetal calf serum. The CHO-K1 cells were grown in Ham's F12, 1% sodium pyruvate, 2% penicillin/streptomycin, 1% fungizone, 10% foetal calf serum and 1% geneticine. After 48 h, transfected cells were harvested, pelleted, resuspended in 0.5 ml of lysis buffer and used immediately for enzymic assay. Fluorescence analysis and Ca^{2+} imaging were performed 24 h after transfection.

Fluorescence analysis

COS-7 cells were seeded on coverslips and transfected with plasmids expressing the different GFP fusions as described above. To analyse the localization of InsP_3 3-kinase B in the ER, the cells were co-transfected with plasmid pDsRed2-ER, which expresses a fusion between the red fluorescent protein DsRed2 and the ER targeting and retention signals of calreticulin. The coverslips were washed with Tris-buffered saline and the cells were fixed for 10 min in a solution of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer. The preparations were mounted in an anti-fading solution (Slow fade light antifade kit from Molecular Probes). Specimens were analysed with a laser scanning confocal microscope (LSM510; Carl Zeiss, Zaventem, Belgium) using a 63 \times objective and light source wavelengths of 488 nm for the excitation of GFP and 543 nm for the excitation of DsRed2.

Table 1 *InsP₃* 3-kinase activities in transfected COS-7 cells

InsP₃ 3-kinase activity was determined in transfected crude cell lysates at 10 μ M *InsP₃* in the presence of 1 mM EGTA or in the presence of 10 μ M free Ca^{2+} and 1 μ M CaM. Activities are shown as means \pm S.D. n.d., not detectable.

	<i>InsP₃</i> 3-kinase activity (nmol \cdot min ⁻¹ \cdot ml ⁻¹)	
	1 mM EGTA	10 μ M Ca^{2+} / 1 μ M CaM
GFP vector	n.d.	n.d.
GFP- <i>InsP₃</i> 3-kinase A	23.00 \pm 0.83	40.46 \pm 2.13
K264A GFP- <i>InsP₃</i> 3-kinase A	0.18 \pm 0.08	0.18 \pm 0.02
GFP- <i>InsP₃</i> 3-kinase B	0.93 \pm 0.31	3.58 \pm 0.24
D897N GFP- <i>InsP₃</i> 3-kinase B	0.2 \pm 0.1	0.9 \pm 0.3
GFP- <i>InsP₃</i> 3-kinase C	12.22 \pm 1.24	5.84 \pm 0.86
K486A GFP- <i>InsP₃</i> 3-kinase C	n.d.	0.23 \pm 0.05

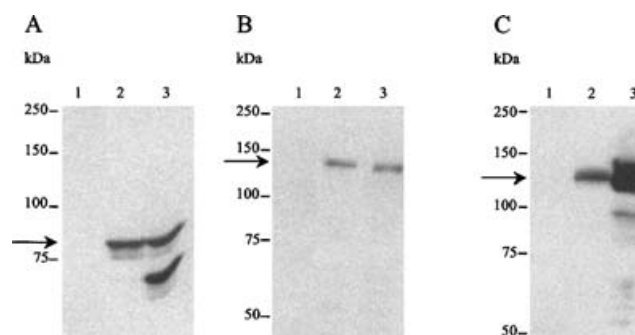
Ca^{2+} imaging

Single-cell $[\text{Ca}^{2+}]_i$ measurements were performed with a laser-scanning MRC-1024 system (Bio-Rad Laboratories) attached to an inverted Nikon Diaphot 300 epifluorescence microscope with a CF Fluor 40 \times (NA = 1.3) oil immersion objective. Briefly, cells were grown in 2 cm² Chamber Slides (Nunc-VWR International sprl, Leuven, Belgium). After removing the culture medium and washing the cells, they were incubated for 30 min with 5 μ M Indo-1-AM dissolved in a modified Krebs solution of the following composition: 135 mM NaCl, 5.9 mM KCl, 1.5 mM CaCl_2 , 1.2 mM MgCl_2 , 11.6 mM Hepes and 11.5 mM glucose (pH 7.3). The cells were then further incubated for at least 1 h in the absence of Indo-1. During the experiment, the cells were superfused continuously from a pipette placed on top of the cell. All the experiments were performed at room temperature (22 °C). Selection of the cells expressing GFP-tagged *InsP₃* 3-kinase isoenzymes was done with the FITC settings of the confocal system. Results were always expressed as ratios of emitted fluorescence. Each scanning was done at 1.3 s, with a scanning box containing 768 \times 512 lines. The signals were not averaged.

RESULTS

The three isoenzymes of *InsP₃* 3-kinase are active enzymes when expressed as GFP-fusion proteins

The cDNAs encoding the three isoenzymes of human *InsP₃* 3-kinase were subcloned in pEGFP to obtain recombinant proteins fused at their N-terminus to GFP. Since *InsP₃* 3-kinases are mainly proteolysed at their N-terminal end, as shown in rat brain or platelets [29,30] but also in COS-7 cells, we preferred to prepare GFP-fusion proteins at the N-terminus of the proteins. This approach yielded proteins that could be analysed by confocal microscopy and fluorescence analysis. This also allowed us to measure $[\text{Ca}^{2+}]_i$ responses to ATP on fluorescent cells and use the adjacent non-fluorescent cells as controls. As shown in Table 1, the three GFP-fused isoforms behaved as expected for the expression of full-length isoforms expressed in bacteria or COS-7 cells [23–25]. Activities of *InsP₃* 3-kinases A–C were determined in the presence of 10 μ M Ca^{2+} /1 μ M CaM and 1 mM EGTA, activities were increased by a factor of 1.8 and 3.8 in the presence of Ca^{2+} /CaM for the A and B isoforms respectively. The activity of *InsP₃* 3-kinase C in the presence of Ca^{2+} /CaM amounted to only half the value obtained in the presence of EGTA. Thus all three enzymes were specifically affected after the

**Figure 1** Western-blot analysis of vector, wild-type and kinase-dead GFP-*InsP₃* 3-kinases A–C

Crude lysates prepared from COS-7 cells transfected with vector (lane 1), wild-type (lane 2) and kinase-dead mutants (lane 3) were subjected to SDS/PAGE and transferred on to nitrocellulose: (A) vector, wild-type 3-kinase A and K264A, (B) vector, wild-type 3-kinase B and D897N, (C) vector, wild-type 3-kinase C and K486A. Immunoblotting was made using anti-GFP antibodies diluted 1:4000. The position of the detected protein is indicated by an arrow. The same extracts were used for enzymic activity (Table 1).

addition of Ca^{2+} /CaM *in vitro*. Control activities measured in cells transfected with empty vector were undetectable. *InsP₃* 3-kinase-dead mutants had much lower or undetectable enzymic activities (Table 1). The expressions of wild-type *InsP₃* 3-kinases A–C and kinase-dead mutants are shown by Western blotting using anti-GFP antibodies (Figure 1). Similar results were obtained by using our *InsP₃* 3-kinases A–C antibodies [23–25]. Extracts from *InsP₃* 3-kinase B-transfected cells always showed lower activities as compared with the two other isoforms, probably reflecting lower efficiency of transfection. When kinase A was transfected with 1.5–15 μ g of DNA, *InsP₃* 3-kinase activity was always stimulated in the presence of Ca^{2+} /CaM by 1.2–1.8-fold (results not shown). When kinase B was transfected with the same amounts of DNA, the degree of stimulation was always higher (4–8-fold). Finally, the results obtained with 3-kinase C showed a slight decrease in the activity in the presence of Ca^{2+} /CaM. Our results therefore indicate that the transfection efficiency (which is clearly not comparable for the three isoforms) did not influence the degree of activation or inhibition we have obtained in our assay conditions and in the presence of Ca^{2+} /CaM.

Intracellular localization of the three *InsP₃* 3-kinase isoenzymes fused N-terminally to GFP

The N-terminal end of *InsP₃* 3-kinase A is responsible for the targeting of the enzyme to F-actin in transfected rat hippocampal cultures [26]. As the N-terminal end of this isoenzyme is rather specific, this suggested that the various isoenzymes of *InsP₃* 3-kinase may be expressed in different areas of the cell as for the inositol 5-phosphatase family [31–33]. We therefore compared the localization of the three isoenzymes fused N-terminally with GFP in COS-7 cells and compared wild-type and N-terminal deletion mutants (Figure 2). The localization of the A isoform to the cytoskeleton is shown in Figures 3(B) and 3(D) compared with vector (pEGFP)-transfected cells (Figure 3A). An N-terminal deletion mutant starting at Leu¹³³ appeared completely cytoplasmic when transfected into COS-7 cells (Figure 3C). Cells stained for F-actin with fluorescent phalloidin are shown in Figure 3(E) and an overlap with *InsP₃* 3-kinase A in Figure 3(F) (in yellow). When cells transfected with the C isoform were analysed, a typical cytoplasmic localization was

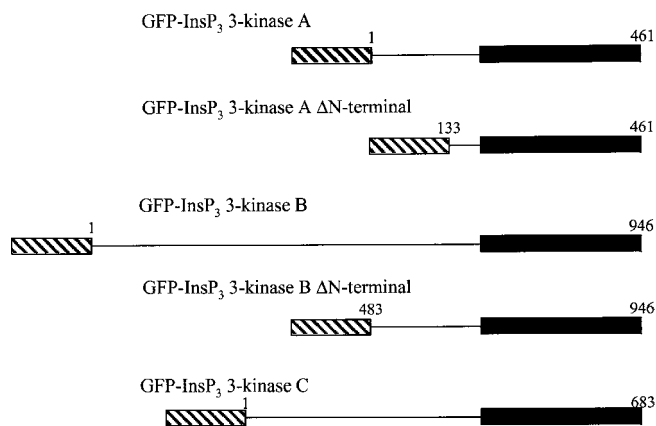


Figure 2 GFP constructs used in the present study

The N-terminal deletion mutant started at Leu¹³³ for *InsP₃* 3-kinase A and at Asp⁴⁸³ for *InsP₃* 3-kinase B. Hatched boxes represent GFP; black boxes represent the conserved catalytic domain of the isoenzymes.

observed in all transfected cells (Figure 4B) as compared with vector-transfected cells (Figure 4A). In contrast, the results obtained with the B isoform were much more complex. Three localizations could be seen, namely cytoskeletal, plasma membrane and ER. Figures 5(B) and 5(G) show the localization of the B isoform at the cytoskeleton together with the plasma membrane; Figure 5(D) suggests a localization at the ER. This localization was confirmed with a targeting structure for the ER, which contained a targeting sequence of calreticulin in red (Figure 5E). The co-localization between the B isoform and the ER-targeted protein is shown in yellow (Figure 5F). Cells stained for F-actin with fluorescent phalloidin are shown in Figure 5(H) and an overlap with *InsP₃* 3-kinase B in Figure 5(I) (in yellow). The

N-terminal deletion mutant of *InsP₃* 3-kinase B starting at Asp⁴⁸³ shows a cytoplasmic localization (Figure 5C).

Effect of overexpression of the three *InsP₃* 3-kinase isoenzymes on Ca^{2+} responses

A series of experiments were performed to compare $[\text{Ca}^{2+}]_i$ responses with 100 μM ATP in *InsP₃* 3-kinase-transfected COS-7 cells. Figure 6 illustrates the response of non-transfected cells to 100 μM ATP in a medium containing 1.5 mM external Ca^{2+} . The cells (100%) showed an initial increase in $[\text{Ca}^{2+}]_i$ levels, which then decreased to a plateau that was maintained as long as the agonist was present. The $[\text{Ca}^{2+}]_i$ levels returned to baseline as soon as ATP was washed away. This pattern is typical for an initial Ca^{2+} release from internal stores, followed by a subsequent phase of influx of external Ca^{2+} . Similar results were obtained in cells transfected with the pEGFP vector alone (Figure 6).

Cells transfected with the *InsP₃* 3-kinase A fused N-terminally to GFP were identified directly by fluorescence. The $[\text{Ca}^{2+}]_i$ responses appeared to be related directly to the GFP fluorescence of the cells (Figure 7; as indicated in grey) and therefore the expression level of each isoenzyme. Highly fluorescent cells did no longer respond to ATP (Figure 7A). When the GFP fluorescence was very low, i.e. just above the background, the response was comparable with that of non-transfected cells. When the fluorescence of the cells was intermediate, one to three Ca^{2+} spikes were obtained in response to ATP. We did not observe any isoenzyme-specific response to ATP when the results were compared among *InsP₃* 3-kinases A–C (Figures 7A–9A). To show the importance of the catalytic activity, control experiments were performed with kinase dead mutants: Ca^{2+} signals in the transfected mutated cells (Figures 7B–9B) were comparable with the pEGFP-transfected cells, in contrast with wild-type enzymes. Similar results were obtained in COS-7 cells and CHO-K1 cells (results not shown). There were no differences when 1 μM instead of 100 μM ATP was used (results not shown).

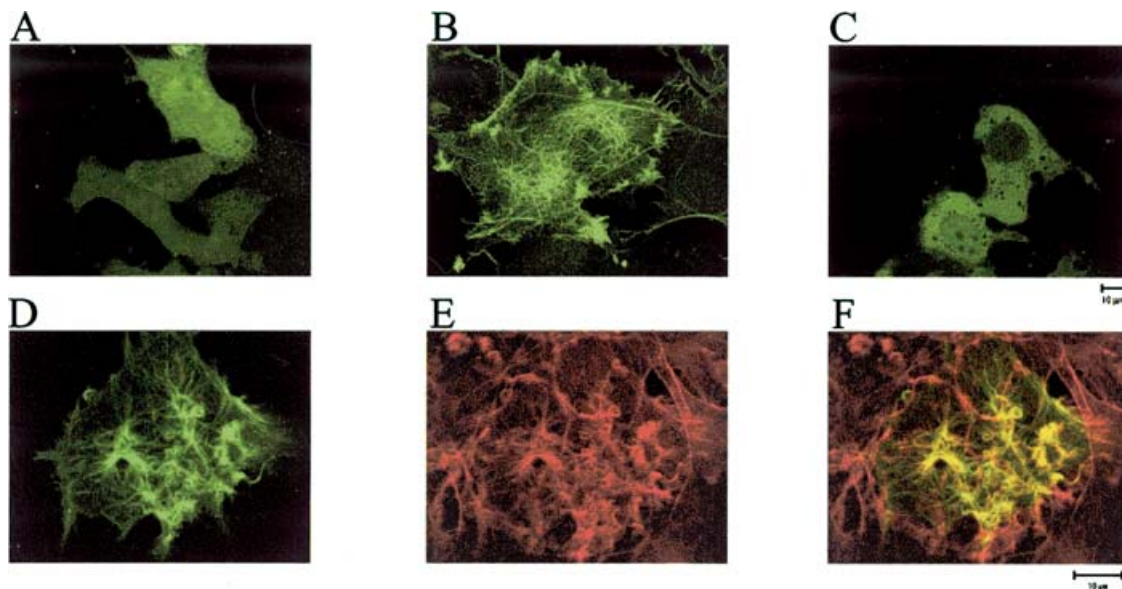


Figure 3 The N-terminal end of *InsP₃* 3-kinase A is required for cytoskeleton localization

COS-7 cells were transfected with *InsP₃* 3-kinase A fused N-terminally to GFP (B, D) or with the GFP vector (A). Cells stained for F-actin with fluorescent phalloidin are shown in red (E) and an overlap with GFP–*InsP₃* 3-kinase A in yellow (F). N-terminal deletion mutant of GFP–*InsP₃* 3-kinase A is shown in (C).

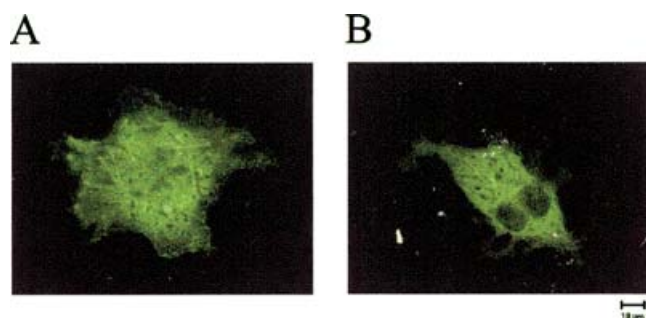


Figure 4 Transfected *InsP₃* 3-kinase C shows a cytoplasmic localization

COS-7 cells were transfected with *InsP₃* 3-kinase C fused N-terminally to GFP (**B**) or with the GFP vector (**A**).

DISCUSSION

Human *InsP₃* 3-kinase isoenzymes are referred to as A, B and C isoforms. Although the biochemistry of the enzymes is well known, the behaviour of each isoform in intact cells is much less studied. This was complicated by the fact that, until recently,

only partial proteins have been used for the B isoform [34] with the possibility that the properties of an enzyme deleted at the N-terminal end would not be comparable with the full-length protein. One major goal of our study was to compare the effect of each of the three isoforms on $[Ca^{2+}]_i$ homoeostasis in intact cells. In a previous study, we compared the $[Ca^{2+}]_i$ responses induced by ATP in CHO-K1 cells transfected either with the prenylated type I *InsP₃* 5-phosphatase or with a mutant that cannot be prenylated. The results support the contention that the ATP-induced increase in *InsP₃* concentration in CHO-K1 cells is essentially restricted to the site of its production near the plasma membrane, where it can be metabolized by the type I *InsP₃* 5-phosphatase [21].

We addressed in the present study the possibility that the three isoenzymes of the *InsP₃* 3-kinase might also affect the $[Ca^{2+}]_i$ response. A series of biochemical experiments and intracellular localization results indicated that the three isoenzymes do not display comparable biochemical properties: (i) the degree of stimulation by Ca^{2+} /CaM on the isoenzyme activities are very different (Table 1) and (ii) the localization of isoenzymes is quite different. The A isoform is attached to the cytoskeleton, the B form is found in the plasma membrane, the cytoskeleton and the ER, whereas the C form is cytoplasmic (Figures 3–5). Our results on the localization of the human C isoform are not in agreement with recent results obtained with the rat C isoform, which is

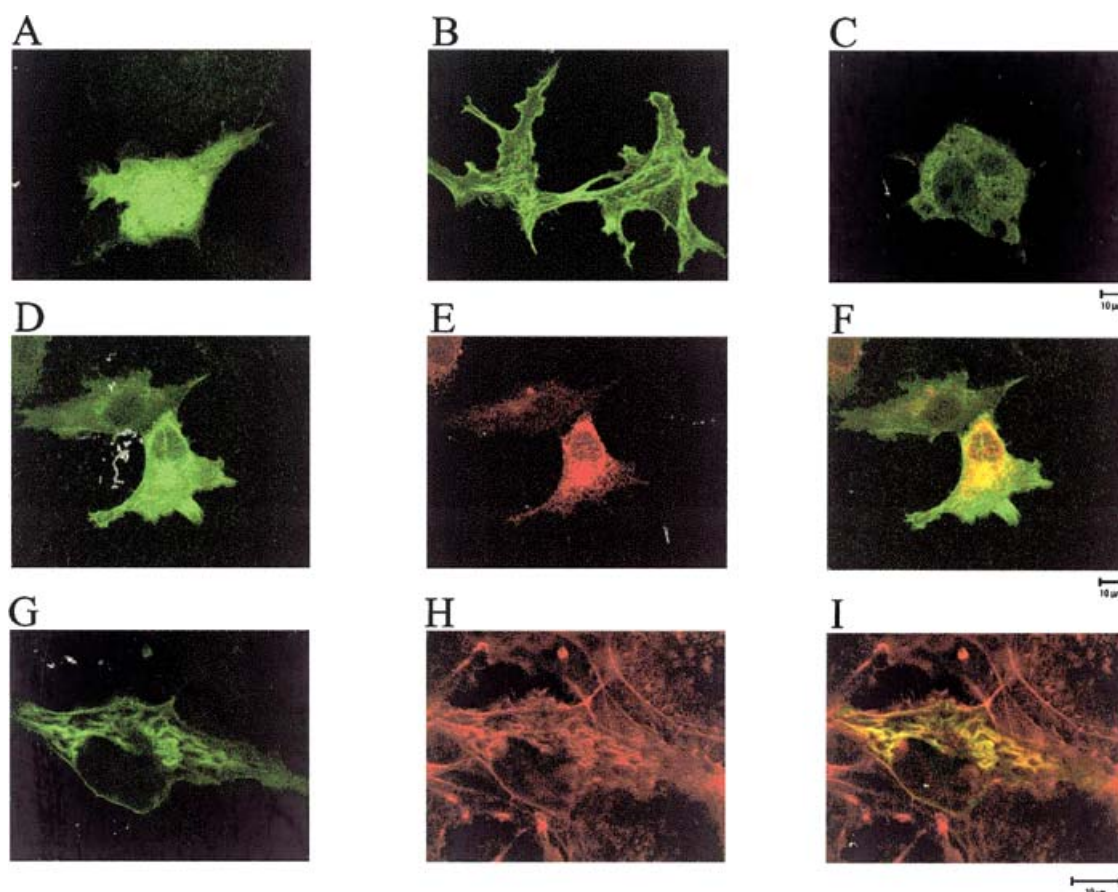


Figure 5 Transfected *InsP₃* 3-kinase B shows a cytoskeletal, plasma membrane and ER localization

COS-7 cells were transfected with *InsP₃* 3-kinase B fused N-terminally to GFP (**B**, **D**, **G**) or with the GFP vector (**A**). The intact enzyme shows a plasma membrane and cytoskeletal localization (**B**) but also an ER localization (**D**). The ER localization of the kinase in green (**D**) is shown together with a fluorescent targeting structure for the ER in red (**E**) and the superposition of the two images in yellow (**F**). Cytoskeletal localization of cells stained for GFP-*InsP₃* 3-kinase B is shown in green (**G**), together with F-actin with phalloidin in red (**H**) and an overlap of the two images in yellow (**I**). The N-terminal deletion mutant of GFP-*InsP₃* 3-kinase B shows a cytoplasmic localization (**C**).

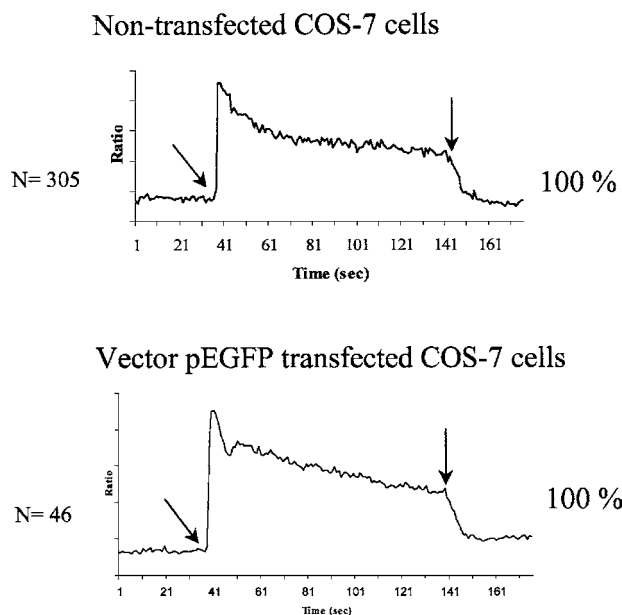


Figure 6 $[Ca^{2+}]_i$ response to $100 \mu M$ ATP in Ca^{2+} -containing solution

Non-transfected COS-7 cells were stimulated by $100 \mu M$ ATP. Totally 305 cells were investigated and show this typical response. The lower panel shows the response of vector pEGFP-transfected cells (46 cells). The time points of ATP addition and wash out are given by the arrows (first and vertical arrows respectively). The results are expressed as ratios of emitted Indo-1 fluorescence.

actively transported in and out of the nucleus when transiently transfected [35]. The reason for this is not understood but could result from the different primary structure of the N-terminal ends in both species, which are only 54 % identical. We have addressed the question whether the localization of the different isoenzymes of $InsP_3$ 3-kinase may affect the $[Ca^{2+}]_i$ response in intact cells. This was done by transfection experiments in COS-7 and CHO-K1 cells. The ATP-induced increase in $[Ca^{2+}]_i$ was affected for the different isoenzymes. The magnitude of the response was related directly to the expression level of each enzyme (tagged with GFP) but did not differ among the isoforms (A, B or C). Previous results obtained in HeLa cells were comparable for the rat partial B isoform [36]. However, the identical response we obtained after A, B and C transfection was unexpected. Therefore it is proposed that the increase in $InsP_3$ 3-kinase activity (A–C) or the resulting increase in V_{max} affects directly the intracellular content of $InsP_3$ in response to ATP, and consequently the Ca^{2+} response, at least in COS-7 or CHO-K1 cells. This is supported by the control experiments performed with the catalytic $InsP_3$ 3-kinase mutants. Mathematical modelling has confirmed the intuitive prediction that the $[Ca^{2+}]_i$ increase in $InsP_3$ 3-kinase activity (as seen for the A and B isoenzymes) can generate $InsP_3$ and Ca^{2+} oscillations [37]. However, the fact that we have obtained identical results with the A and C isoforms stimulated and inhibited by Ca^{2+} respectively (Table 1) argues in favour of a general reduction of $InsP_3$ levels so that the $InsP_3$ concentration is below the threshold to generate oscillations. Alternatively, Ca^{2+} oscillations observed at low expression levels of each isoform can be generated in the presence of a constant level of $InsP_3$ controlling the presence and frequency of Ca^{2+} oscillations [38]. Based on the results we have obtained in the present study, we suggest that our previous results obtained in type I inositol 5-phosphatase-transfected CHO-

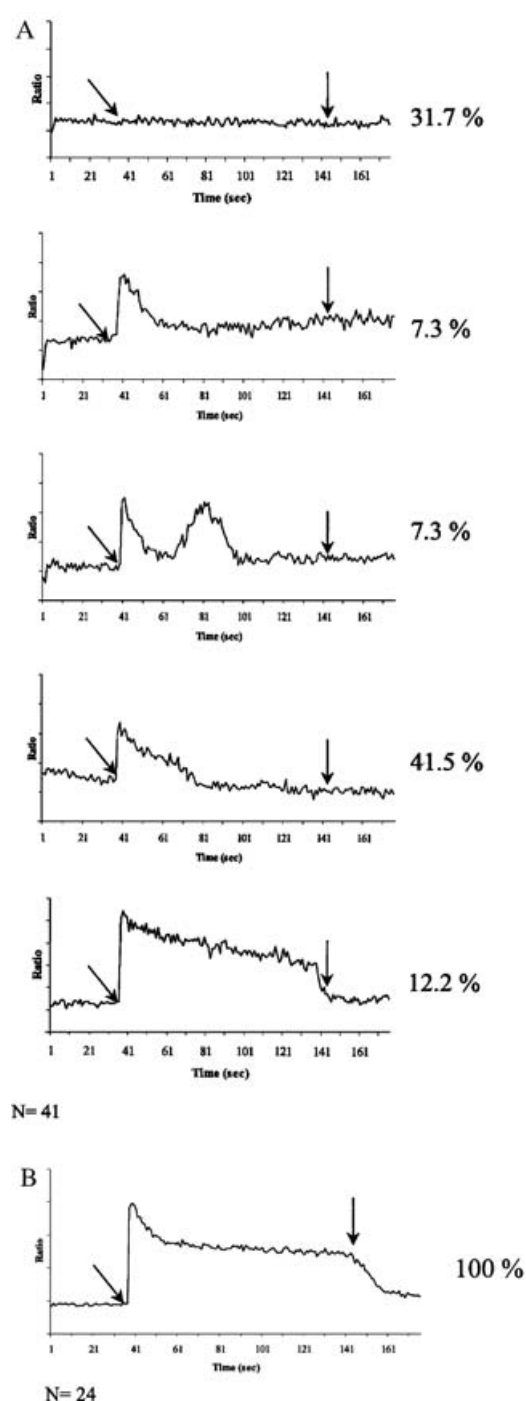


Figure 7 $[Ca^{2+}]_i$ responses to $100 \mu M$ ATP in Ca^{2+} -containing solution of $InsP_3$ 3-kinase A-transfected cells

(A) COS-7 cells were transfected with GFP- $InsP_3$ 3-kinase A and stimulated with $100 \mu M$ ATP. The response of each individual cell is correlated directly with the relative level of GFP fluorescence (in grey) and indicated as a percentage of the total number of cells investigated. The time points of ATP addition and wash out are given by the arrows. (B) COS-7 cells transfected with kinase-dead mutant K264A GFP- $InsP_3$ 3-kinase A. The results are expressed as ratios of emitted Indo-1 fluorescence.

K1 cells result from a large reduction in $InsP_3$ levels due to an increased phosphatase activity [21]. This was not the case in cells transfected with an enzyme with a mutated prenylation site,

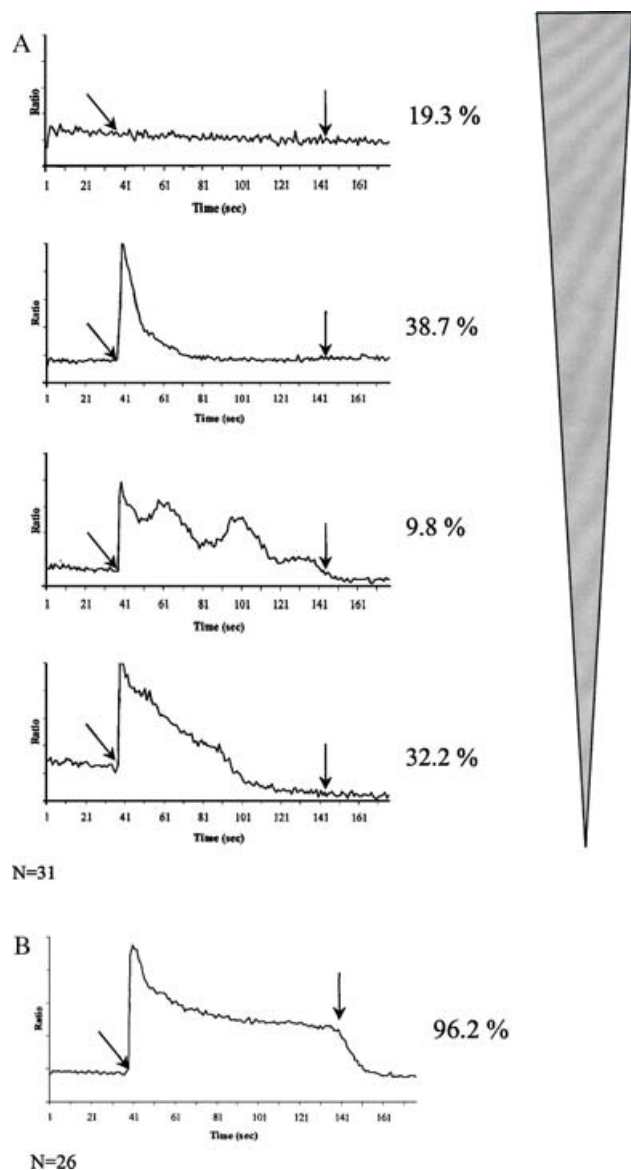


Figure 8 $[Ca^{2+}]_i$ responses to 100 μ M ATP in Ca^{2+} -containing solution of $InsP_3$ 3-kinase B-transfected cells

(A) COS-7 cells were transfected with GFP- $InsP_3$ 3-kinase B and stimulated with 100 μ M ATP. The response of each individual cell is correlated directly with the relative level of GFP fluorescence (in grey) and indicated as a percentage of the total number of cells investigated. (B) COS-7 cells transfected with kinase-dead mutant D897N GFP- $InsP_3$ 3-kinase B. The time points of ATP addition and wash out are given by the arrows. The results are expressed as ratios of emitted Indo-1 fluorescence.

suggesting that in intact cells the wild-type and mutant enzymes had different phosphatase activities.

Previous results of Banting and co-workers [27] have indicated that a truncated form of rat $InsP_3$ 3-kinase B (673 amino acids) exists both as a peripheral membrane protein associated tightly with the ER network, and as a cytosolic protein. The results were obtained after transfection in COS-7 cells. The results obtained in the present study using a full-length protein confirm ER localization but also show the presence of this isoform in the cytoskeleton of transfected COS-7 cells. It is tempting to speculate that the expression of a full-length protein of 946 amino acids could explain the discrepancy between the two results. The targeting

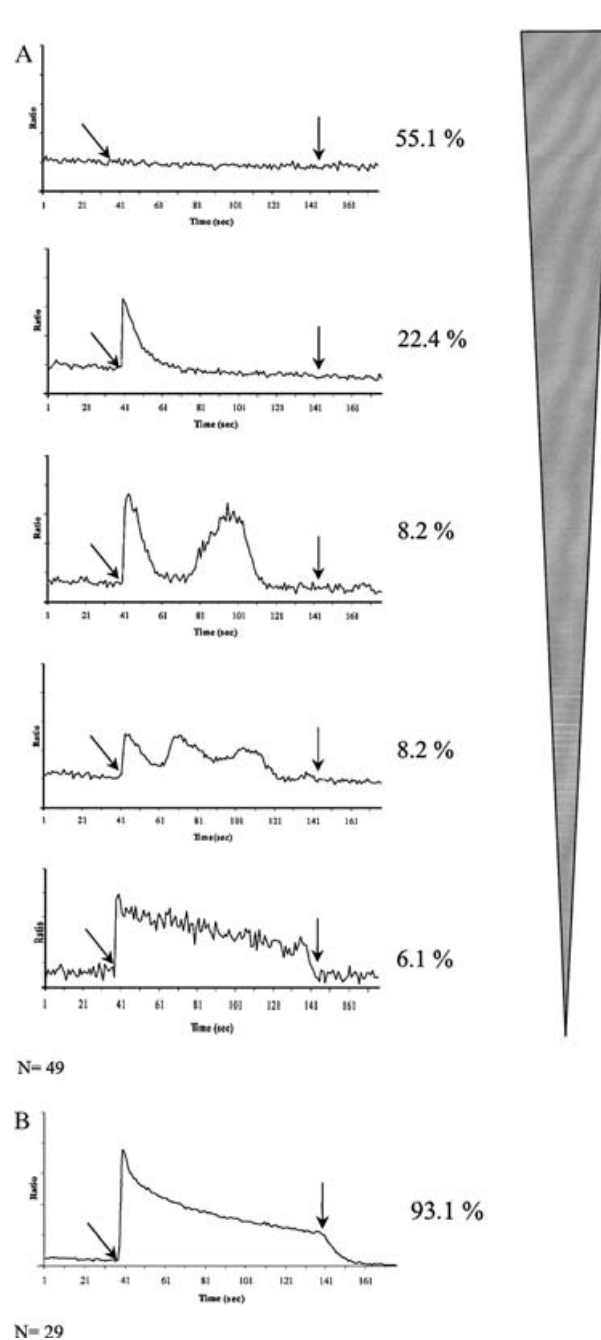


Figure 9 $[Ca^{2+}]_i$ responses to 100 μ M ATP in Ca^{2+} -containing solution of $InsP_3$ 3-kinase C-transfected cells

(A) COS-7 cells were transfected with GFP- $InsP_3$ 3-kinase C and stimulated with 100 μ M ATP. The response is correlated directly with the relative level of GFP fluorescence (in grey) and indicated as a percentage of the total number of cells investigated. (B) COS-7 cells transfected with kinase-dead mutant K486A GFP- $InsP_3$ 3-kinase C. The time points of ATP addition and wash out are given by the arrows. The results are expressed as ratios of emitted Indo-1 fluorescence.

of $InsP_3$ 3-kinase A to F-actin has been proposed to result from the presence of an N-terminal proline-rich stretch followed by an 18-amino acid α -helix predicted in the secondary structure of the A isoform [26]. Secondary structure analysis of $InsP_3$ 3-kinase B also shows the presence of an α -helix by two Internet-based programs (SOPM and nnPredict, <http://us.expasy.org>). This

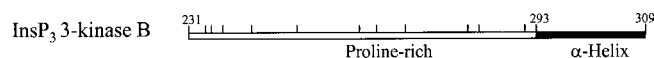


Figure 10 Proline-rich sequences in the N-terminal region of InsP₃ 3-kinase B

The sequence of amino acids 231–309 of human InsP₃ 3-kinase B shows an α -helix preceded by a proline-rich region. Each vertical line indicates the position of a proline residue.

α -helix is preceded by several prolines; which could explain the targeting of InsP₃ 3-kinase B to the cytoskeleton. The percentage of proline residues in this region is 17% (Figure 10).

In conclusion, we suggest that both metabolizing enzymes [InsP₃ 5-phosphatase and 3-kinase] may control InsP₃ levels at the single-cell level. The localization of InsP₃ 3-kinase isoenzymes is intriguing but could be related to co-ordinated regulation between InsP₃ receptor(s) and its metabolism due to similar localization, e.g. in the ER and in the plasma membrane [39,40]. Hirose et al. [41] proposed that the oscillations in InsP₃ concentration might depend on Ca²⁺ having both enhancing and inhibitory effects on phospholipase C. Recently, InsP₃ oscillations have been measured in single CHO cells. The predominant underlying mechanism resulted from a negative feedback loop whereby protein kinase C inhibited InsP₃ generation [42]. Our results suggest that InsP₃ oscillations at least in some cells could be generated due to the stimulation of InsP₃ 3-kinase activity by Ca²⁺ for the A and B isoenzymes. In this respect, the different sensitivity of the two isoenzymes to Ca²⁺ could generate different oscillation patterns of InsP₃ and in turn specific Ca²⁺ responses. Experiments could be performed in future to test this hypothesis.

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